JOURNAL OF VIROLOGY, Aug. 2000, p. 6992–7004 Vol. 74, No. 15
0022-538X/00/$04.00+0

Genetic Diversity among Lassa Virus Strains

MICHAEL D. BOWEN,† PIERRE E. ROLLIN, THOMAS G. KSIAZEK, HEATHER L. HUSTAD, DANIEL G. BAUSCH, AUSTIN H. DEMBY,‡ MARY D. BAJANI,§ CLARENCE J. PETERS, AND STUART T. NICHOL*

Special Pathogens Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333

Received 7 January 2000/Accepted 4 May 2000

The arenavirus Lassa virus causes Lassa fever, a viral hemorrhagic fever that is endemic in the countries of Nigeria, Sierra Leone, Liberia, and Guinea and perhaps elsewhere in West Africa. To determine the degree of genetic diversity among Lassa virus strains, partial nucleoprotein (NP) gene sequences were obtained from 54 strains and analyzed. Phylogenetic analyses showed that Lassa viruses comprise four lineages, three of which are found in Nigeria and the fourth in Guinea, Liberia, and Sierra Leone. Overall strain variation in the partial NP gene sequence was found to be as high as 27% at the nucleotide level and 15% at the amino acid level. Genetic distance among Lassa strains was found to correlate with geographic distance rather than time, and no evidence of a "molecular clock" was found. A method for amplifying and cloning full-length arenavirus S RNAs was developed and used to obtain the complete NP and glycoprotein gene (GP1 and GP2) sequences for two representative Nigerian strains of Lassa virus. Comparison of full-length gene sequences for four Lassa virus strains representing the four lineages showed that the NP gene (up to 23.8% nucleotide difference and 12.0% amino acid difference) is more variable than the glycoprotein genes. Although the evolutionary order of descent within Lassa virus strains was not completely resolved, the phylogenetic analyses of full-length NP, GP1, and GP2 gene sequences suggested that Nigerian strains of Lassa virus were ancestral to strains from Guinea, Liberia, and Sierra Leone. Compared to the New World arenaviruses, Lassa and the other Old World arenaviruses have either undergone a shorter period of diverisification or are evolving at a slower rate. This study represents the first large-scale examination of Lassa virus genetic variation.

Viruses of the genus *Arenavirus*, family *Arenaviridae*, are enveloped viruses with a genome consisting of two single-stranded RNA species designated small (S) and large (L). Each segment contains two nonoverlapping genes arranged in an ambisense orientation (56). The viral polymerase (L protein) gene is encoded at the 3′ end of the L RNA in the genome-complementary sense, whereas the Z protein is encoded at the 5′ end of the L RNA in the genomic sense. In a similar fashion, the nucleoprotein (NP) gene is encoded at the 3′ end of the S RNA, whereas the glycoprotein precursor (GPC) is encoded at the 5′ end of the S RNA. The GPC is posttranslationally cleaved into the envelope glycoproteins GP1 and GP2 (56). The arenaviruses have been divided into two groups, the New World arenaviruses and the Old World arenaviruses (18).

Lassa fever, a viral hemorrhagic fever that is endemic in the West African countries of Nigeria, Sierra Leone, Liberia, and Guinea, is caused by Lassa virus, an Old World arenavirus. *Mastomys* sp., a poorly defined species complex of rodents (32), is the reservoir host of Lassa virus. Humans presumably become infected through contact with infected rodent excreta, tissues, or blood (46, 49, 60). Person-to-person transmission of Lassa fever can also occur (22, 49). An estimated 100,000 to 300,000 infections and approximately 5,000 deaths occur annually (47). Between January 1996 and March 1997, over 1,000 cases of Lassa fever with 148 deaths were reported from eastern Sierra Leone (3).

Despite the public health importance of Lassa fever, little is known about the genetic diversity or relationships of Lassa viruses found in various parts of West Africa. Antigenic differences have been detected between Nigerian Lassa virus isolates and isolates originating in Sierra Leone, Liberia, and Guinea (39, 54). RNA fingerprinting and comparison of short sequences have demonstrated genetic differences among single strains from Sierra Leone and Liberia and two strains from Guinea (31, 59). Genomic sequence data for only three strains have been deposited in sequence databases: the prototype LP strain from northeastern Nigeria (partial NP gene sequence) (11, 13), the GA391 strain from central Nigeria (complete S RNA sequence) (17), and the Josiah strain from Sierra Leone (complete S and L RNA sequences (5, 19, 45). Considerable sequence dissimilarity (greater than 22% nucleotide and 11% amino acid sequence divergence) in a region of the NP gene was noted not only between the Nigerian strains and the Sierra Leone Josiah strain but also between the two Nigerian strains (11). The genetic distance between the Nigerian strains (LP and GA391) was unexpected, since their origins of isolation are only about 500 km apart and separated by only an 8-year interval. Although there are no currently established criteria for differentiating arenavirus species based on genetic data, the

* Corresponding author. Mailing address: Special Pathogens Branch, Mailstop G14, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333. Phone: (404) 639-1115. Fax: (404) 639-1118. E-mail: stn1@cdc.gov.

† Present address: Bioterrorism Preparedness and Response Program, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333.

‡ Present address: Tuberculosis/Mycobacteriology Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333.

§ Present address: Emerging Bacterial and Mycotic Disease Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333.

TABLE 1. Lassa virus strains examined in this study

No.	Type	Date[a]	Country	Region	Host	Reference	Identical sequence
806828	Isolate	1981	Guinea	Macenta	Human	39	
807868	Isolate	1996	Guinea	Faranah	Human		
808255	Isolate	1996	Guinea	Faranah	Rodent		
807992	Isolate	1997	Guinea	Kissedougou	Human		
807998	Isolate	1997	Guinea	Nzerekore	Human		
803204	Isolate	1972	Liberia	Zorzor	Human	50	803201
803201	Isolate	1972	Liberia	Zorzor	Human	50	803204
803791	Isolate	1980	Liberia	Zorzor	Human	23	
803792	Isolate	1980	Liberia	Zorzor	Human	23, 39	
803793	Isolate	1980	Liberia	Zorzor	Human	23, 39	
806829	Isolate	1981	Liberia	Zorzor	Human	23, 39	
803796	Isolate	1981	Liberia	Phebe	Human	23	
807977	Isolate	1981	Liberia	Zorzor	Human	23, 39	
803203	Isolate	1972	Liberia	Zorzor	Human		
803213	Isolate	1974	Nigeria	Onitsha	Human	8	803214
803214	Isolate	1974	Nigeria	Onitsha	Human	8	803213
803210	Isolate	1975	Nigeria	Vom	Human	23	
803208	Isolate	1975	Nigeria	Zonkwa	Human	49	
803212	Isolate	1976	Nigeria	Vom	Human		807975
807975	Isolate	1976	Nigeria	Vom	Human		803212
803787	Isolate	1981	Nigeria	?	Human	39	
806316	Isolate	1989	Nigeria	Ekpoma	Human	22	
806319	Isolate	1989	Nigeria	Aba	Human	22	806320
806320	Isolate	1989	Nigeria	Aba	Human	22	806319
806321	Isolate	1989	Nigeria	Owerri	Human	22	
806322	Isolate	1989	Nigeria	Owerri	Human	22	
806791	Isolate	1993	Nigeria	Jos	Human		
808031	Isolate	1994	Nigeria	Ekpoma	Human		
9608911	Serum	1996	Nigeria	Jos	Human		
803211	Isolate	1972	Sierra Leone	Panguma	Human		
803209	Isolate	1975	Sierra Leone	Segbwema	Human		
803205	Isolate	1976	Sierra Leone	Segbwema	Human		
803206	Isolate	1976	Sierra Leone	Panguma	Human		
807974	Isolate	1976	Sierra Leone	Mobai	Human	65	
807976	Isolate	1977	Sierra Leone	Tongo	Human		
806827	Isolate	1977	Sierra Leone	Gondama	Human		
801100	Isolate	1978	Sierra Leone	Tongo	Rodent	41	
801101	Isolate	1978	Sierra Leone	Tongo	Rodent	41	
801102	Isolate	1978	Sierra Leone	Tongo	Rodent	41	
801103	Isolate	1978	Sierra Leone	Tongo	Rodent	41	
801104	Isolate	1978	Sierra Leone	Konia	Rodent		
801105	Isolate	1978	Sierra Leone	Konia	Rodent		801107
801106	Isolate	1978	Sierra Leone	Konia	Rodent		
801107	Isolate	1978	Sierra Leone	Konia	Rodent		801105
801108	Isolate	1978	Sierra Leone	Konia	Rodent		
801109	Isolate	1978	Sierra Leone	Konia	Rodent		
801618	Isolate	1979	Sierra Leone	Konia	Human		
802662	Isolate	1980	Sierra Leone	Niahun	Human		
803972	Isolate	1982	Sierra Leone	Mano	Human		
807875	Isolate	1982	Sierra Leone	?	Rodent		
806843	Isolate	1993	Sierra Leone	Tongo	Human		
9607290	Serum	1996	Sierra Leone	Segbwema	Human		
9607300	Serum	1996	Sierra Leone	Tongo	Human		
9607302	Serum	1996	Sierra Leone	Tongo	Human		

[a] Date virus isolated or serum drawn.

apparent diversity of Lassa viruses has prompted speculation that Lassa virus per se may actually be a complex of closely related arenaviruses (44). The purpose of this study was to perform a large-scale examination of the genetic diversity of Lassa viruses across the currently known geographic range of Lassa fever. Attempts were made to incorporate into the study a selection of geographic and temporal variants as well as strains isolated from *Mastomys* rodents and humans. The objectives of the study were to further the understanding of Lassa virus genetics and evolution, provide data for establishing criteria for genetic classification of arenavirus species, and provide insights into the epidemiology of Lassa fever.

MATERIALS AND METHODS

Virus strains. Lassa virus isolates and clinical samples were obtained from the virus collection of the Special Pathogens Branch, Centers for Disease Control and Prevention, Atlanta, Ga. The virus strains examined in this study are listed in Table 1, and their origins are shown in the maps in Fig. 1. All work with potentially infectious materials was carried out in the biosafety level 4 laboratories of the Special Pathogens Branch. Virus stocks for RNA extraction were grown in monolayers of Vero E6 cells (ATCC CRL-1586) cultured in Eagle's

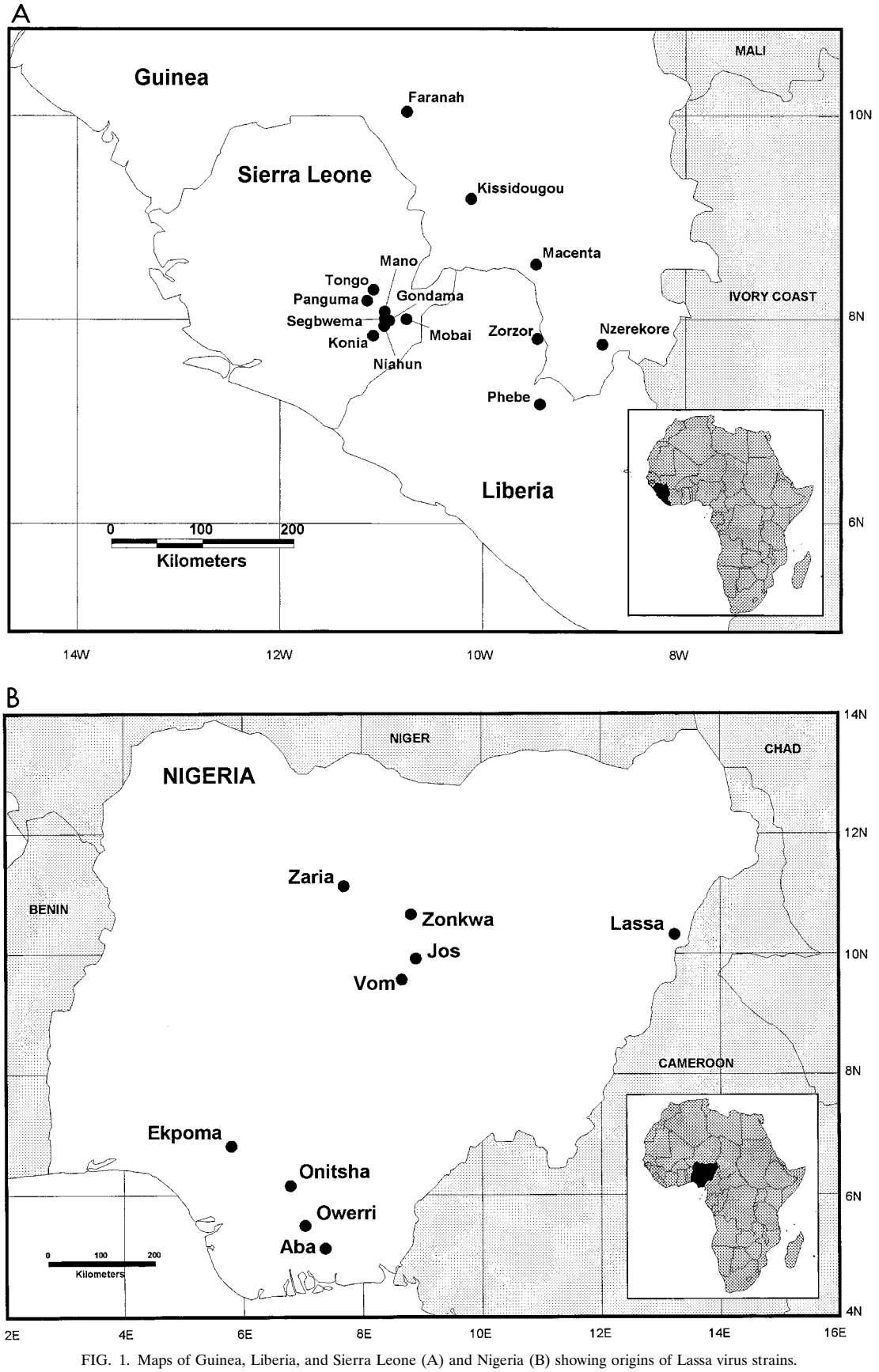

FIG. 1. Maps of Guinea, Liberia, and Sierra Leone (A) and Nigeria (B) showing origins of Lassa virus strains.

minimal essential medium supplemented with 2% fetal bovine serum, L-glutamine, antibiotics, and Fungizone (GIBCO BRL). The clinical specimens examined in this study consisted of serum samples from Lassa fever patients.

RNA extraction. RNA was extracted from cell culture supernatant harvested from infected cell cultures or directly from virus stocks. A 300-μl aliquot of cell culture supernatant was mixed with 1 ml of TriPure reagent (Boehringer Mannheim) and 200 μl of chloroform-isoamyl alcohol (24:1); RNA was extracted following the manufacturer's instructions. When the source of RNA was human serum rather than a virus stock, RNA was recovered from the aqueous layer with an RNaid kit (Bio101) instead of by isopropanol precipitation.

RT-PCR. Reverse transcription (RT)-PCR was initially performed with an oligonucleotide primer pair described previously (11) that targeted a region near the 3′ end of the NP gene of Old World arenaviruses. The genome-complementary primer 1010C (5′-TCIGGIGAIGGITGGCC-3′ [I, inosine]) was used in conjunction with the genomic-sense primer OW1696R (5′-AIATGAIGCAGTC CAIIAGTGCACAGTG-3′). RT-PCR was carried out with an Access RT-PCR kit (Promega) and a Perkin-Elmer 9600 thermocycler. Each RT-PCR mixture contained 1× avian myeloblastosis virus (AMV)-*Tfl* reaction buffer, 1 μM primer 1010C, 1 μM primer OW1696R, 500 μM $MgSO_4$, 200 μM deoxynucleoside triphosphates, 5 U of AMV reverse transcriptase, 5 U of *Tfl* DNA polymerase, and 10% of the RNA from a single RNA extraction. RT was carried out for 1 h at 42°C, and the reaction mixtures were heated for 2 min at 94°C. The reaction mixtures were then subjected to 40 cycles of a temperature profile consisting of 94°C for 30 s, 45°C for 1 min, and 68°C for 2 min, followed by a final extension of 68°C for 7 min. Some Lassa virus stocks failed to amplify with primers 1010C and OW1696R, and therefore primer OW1696R was redesigned to produce primer 1696RV2 (5′-AIATGATGCAGTCCAIIAGIGCACA-3′). This primer was then used in conjunction with primer 1010C to obtain RT-PCR products as described previously.

Cloning of complete S RNA sequences. To obtain the complete S RNA sequences of Lassa virus isolates, virus RNA was first denatured by incubation with 10 mM methyl mercury hydroxide for 10 min at room temperature. Excess methyl mercury hydroxide was then bound by adding 2 μl of 700 mM 2-mercaptoethanol and 4 U of RNasin (Promega) and incubating the mixture for 15 min at room temperature. RT was then carried out in a 30-μl reaction mixture containing 1 μM primer M13-19C (5′-TGTAAAACGACGGCCAGTGCGCA CAGTGGATCCTAGGC-3′), 1× AMV reverse transcriptase buffer (Life Sciences Inc.), 1 mM deoxynucleoside triphosphates, 20 U of RNasin, and 30 U of AMV reverse transcriptase XL (Life Sciences Inc.) After the mixture was incubated for 2 h at 42°C, 30 μl of 2× RNA hydrolysis solution (0.4 N NaOH, 40 mM EDTA) was added and the reaction mixture was incubated for 30 min at 65°C. The cDNAs were recovered by ethanol precipitation with ammonium acetate.

Virus S segment cDNAs were then amplified using the Expand high-fidelity PCR system (Boehringer Mannheim) in a 100-μl reaction mixture containing 1× Expand buffer without $MgCl_2$, 600 nM primer M13-19C, 2 mM $MgCl_2$, 2% dimethyl sulfoxide, 1 μl cDNA suspension, and 3.5 U of Expand enzyme mixture. Amplification was carried out on a Perkin-Elmer 9600 thermocycler, using a profile that consisted of an initial 2 min at 94°C followed by 30 cycles of 94°C for 15 s, 45°C for 30 s, and 72°C for 2 min, with the extension time increased in 20-s increments from cycles 11 through 30, followed by a 7-min hold at 72°C. The amplified products were phenol-chloroform extracted, recovered by ethanol precipitation with ammonium acetate, and then 3′ end tailed with dATP in a 50-μl reaction mixture containing 1× PCR buffer without $MgCl_2$ (Boehringer Mannheim), 800 nM dATP, 3 mM $MgCl_2$, and 5 U of *Taq* polymerase (Boehringer Mannheim). The reaction mixture was incubated for 1 h at 75°C and then electrophoresed on a 0.8% agarose gel in Tris-acetate-EDTA buffer. The amplified products were located by staining the gel with 0.02% methylene blue dye, sliced from the gel, purified from the gel slices using a Sephaglas Bandprep kit (Pharmacia Biotech), and ligated to the pCRII plasmid vector (Invitrogen). The ligation mixture was then used to transform MAX EFFICIENCY DH5α competent *Escherichia coli* (Life Technologies Inc.), and plasmids were recovered by standard minipreparation procedures. Potential S RNA clones were identified by restriction digest analysis of plasmids using *Eco*RI.

Sequence determination. To obtain sequence data directly from RT-PCR products, the remaining RT-PCR mixture was electrophoresed on a 1% agarose gel in Tris-acetate-EDTA buffer. After ethidium bromide staining, specific PCR products were located by UV translumination, sliced from the gel, and purified from the gel slices using a Sephaglas Bandprep kit (Pharmacia Biotech). Dye terminator cycle-sequencing reactions were carried out using 5 to 50% of the gel-purified product (depending on product yield), ABI PRISM with AmpliTaq DNA polymerase FS or BigDye dye terminator cycle-sequencing ready-reaction kits (Applied Biosystems), and 3.3 pM primer 1010C, OW1696R, or 1696RV2. The extension products were purified using Centri-sep spin columns (Princeton Separations) and sequenced on a model 377 automated DNA sequencer (Applied Biosystems). Sequences were obtained from both strands of each RT-PCR product for verification. S RNA clones were sequenced initially using pCRII plasmid-specific primers. Additional S RNA sequence was obtained by sequence "walking" through the plasmid insert using the following Lassa virus S RNA-specific primers designed from newly determined sequence: NP479C (5′-CGG GTGTITACATGG-3′), NP514C (5′-CTTGAICAGAGGAGGGC-3′), NP623R (5′-TCTG[G/C]ATTTTTIAC[A/G]TCCCA-3′), NP1277R (5′-ATCTCCACIG GGTCTTC-3′), NP1555C (5′-AAGTTTGAAAATGCTGTITGGGA-3′), GPC2158R (5′-GAACAGCAAGCIGACAA[C/T]ATGAT-3′), GPC2283C (5′-GG[A/T]ATICCCATGATGTC-3′), GPC2778C (5′-CCCCA[A/G]GCCAT [C/T]CTCATAA-3′), and GPC2871R (5′-ATGAGTTGTGATTTCAATGG-3′) (I, inosine; [N/N] indicates mixed bases). In some cases, RT-PCR products were not sequenced directly but cloned into the TA cloning vector pCRII (Invitrogen) and then sequenced with pCRII plasmid-specific primers. In both cases, the sequence was read from both strands of a minimum of three independent clones. Chromatogram analysis and sequence compilation were performed with Sequencher 3.0 software (Gene Codes). RNA secondary-structure predictions were performed with the MFold and PlotFold programs of the Wisconsin Package version 9.1-UNIX (Genetics Computer Group, Inc.), and gene sequences were translated with the Translate program of the Wisconsin Package.

Phylogenetic and statistical analyses. Alignments were performed with the PileUp program of the Wisconsin Package, with a gap creation penalty of 2.0 and a gap extension penalty of 0.2, followed by manual adjustment or with Clustal X version 1.63b. Maximum-parsimony (MP), maximum-likelihood (ML), and minimum-evolution (ME) phylogenetic analyses were performed with PAUP* version 4.0b1 (58). Quartet puzzling (QP) analyses were carried out with PUZZLE 4.0.1 (57). Statistical analyses were performed with SPSS 8.0.1 (SPSS Inc.) and MatMan version 1.0 (Matman, Noldus Information Technology, Wageningen, The Netherlands, 1998).

Nucleotide sequence accession numbers. The partial NP sequences obtained from 54 Lassa virus strains were deposited in GenBank under accession no. AF182219 through AF182272.

RESULTS

Lassa virus strains comprise four genetic lineages. Partial NP sequences were obtained from 54 Lassa virus strains and yielded 49 unique sequences. RT-PCR primers 1010C and OW1696R were reactive with all strains from Sierra Leone, Liberia, and Guinea but failed to amplify some Nigerian strains. Primer OW1696R was redesigned to produce primer 1696RV2, which reacted with all Lassa virus strains when used with primer 1010C. This primer pair was used for subsequent characterization of Lassa virus strains. The sequences were 628 nucleotides (nt) long when amplified with primers 1010C and OW1696R and 631 nt long if amplified with primers 1010C and 1696RV2. Nucleotides 1 to 628 of the Lassa virus partial NP sequences were aligned with the corresponding genomic region of other Old World arenaviruses and three New World arenaviruses, which served as outgroup taxa. Phylogenetic analysis of the unweighted data set using MP optimality yielded a single most parsimonious tree of 2,744 steps (Fig. 2). The robustness of the resulting phylogeny was evaluated by bootstrap analysis (21) and calculation of Bremer support indices (BSI) (12). Lassa virus strains occupied a single strongly supported (bootstrap support 90%; BSI value, >5) large clade composed of four geographic lineages, which are designated I, II, III, and IV (Fig. 2). The prototype LP strain of Lassa virus is the sole occupant of lineage I. The LP strain originates from the village of Lassa, located in northeastern Nigeria (24) (Fig. 1B), and it is the only strain in the study originating from this region. Lineage II is a strongly supported group (Fig. 2) that contains strains from sites in southern central Nigeria: Aba, Ekpoma, Onitsha, and Owerri (8, 22) (Fig. 1B). Lineage III (Fig. 2) contains strains from sites in northern central Nigeria: Jos, Vom, Zonkwa, and Zaria (23, 49; D. J. Grundy, E. T. Bowen, and G. Lloyd, Letter, Lancet **ii:**649–650, 1980) (Fig. 1B). It includes the GA391 strain of Lassa from Zaria, which was genetically characterized by Clegg and coworkers (17), and strain 803787 (Table 1), which was serologically characterized by Jahrling and coworkers (39), but its geographic origin within Nigeria was unrecorded. The monophyly of this group of Lassa isolates is strongly supported by bootstrap analysis and BSI, with the exception of strain 9608911. This strain occupies the most basal position in the lineage, but bootstrap support for monophyly of this strain with the others in lineage III is poor (37%). The BSI for this association, however, is two steps above parsimony, thus providing some support for the monophyly of lineage III.

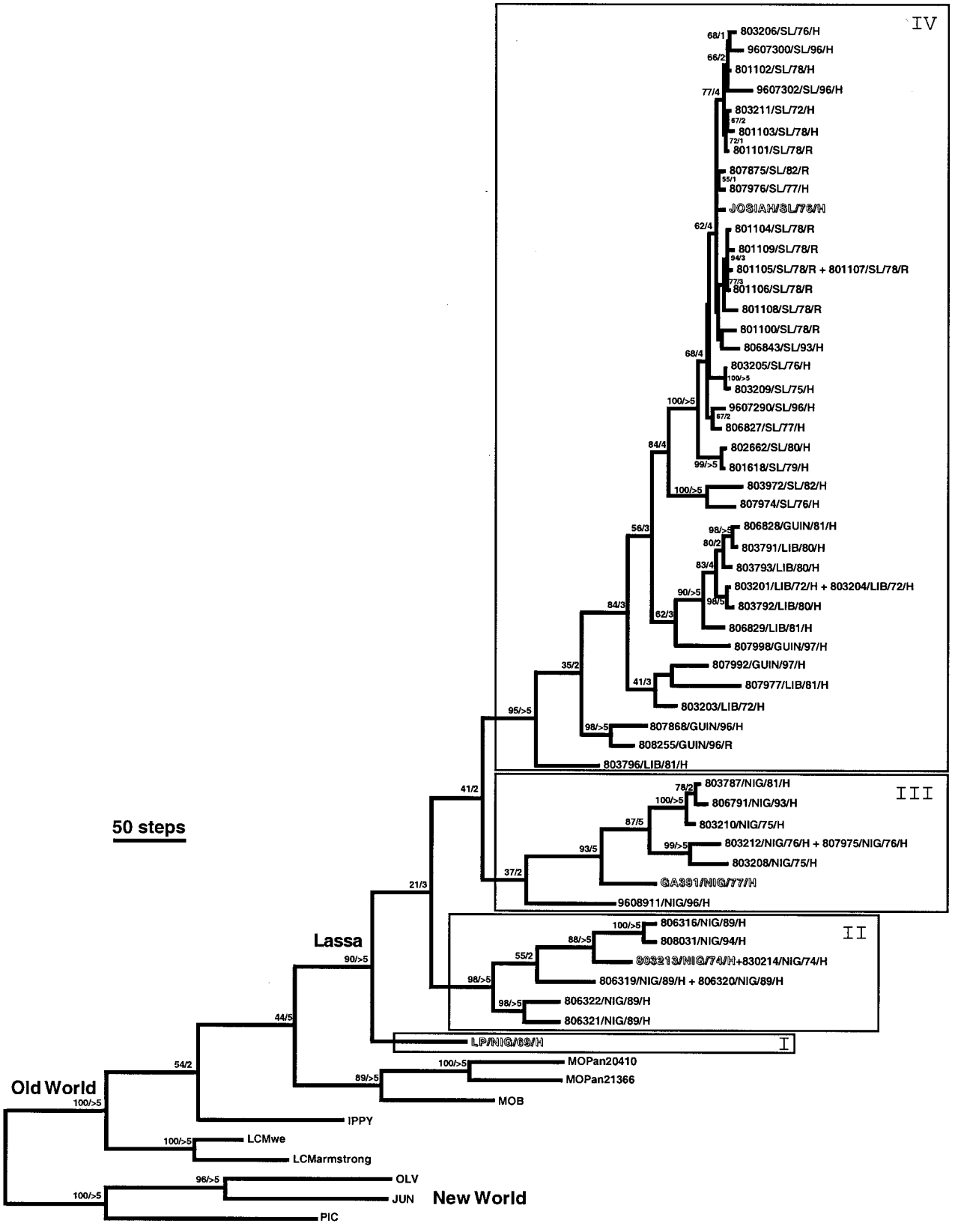

FIG. 2. Phylogenetic tree inferred using MP criteria that shows relationships among Lassa virus strains. Lassa virus partial NP gene sequences were aligned with the corresponding regions of the Josiah (GenBank accession number J04324 [5]), GA391 (X52400 [17]), and LP (U80004 [11]) strains of Lassa virus as well as the corresponding regions of OLV virus (U34248 [9]), PIC virus (K02734 [4, 6]), Junin virus strain XJ (U80009 [11]), LCM virus strains Armstrong (M20869 [55]) and WE (M22138 [53]), Ippy virus (U80003 [11]), Mopeia virus strains AN20410 (U80005 [11]) and AN21366 (M33879 [63]), and Mobala virus strain 3076 (AF012530 [11]). Phylogenetic analysis of the partial NP gene data set was carried out using PAUP* 4.0, with characters optimized by accelerated transformation and gaps treated as missing data. The data set contained 667 characters, of which 423 were parsimony informative. The large number of taxa necessitated the use of a heuristic search that was performed using stepwise addition, with random addition (10 replicates) and tree bisection-reconnection branch swapping. The unweighted analysis yielded a single most parsimonious tree of 2,744 steps. The numbers adjacent to each node are percentage bootstrap support (500 replicates)/BSIs. Horizontal branch lengths are indicated by the scale bar. The four Lassa virus lineages are labeled I through IV. Representative strains from each of the four lineages used for further genetic analysis are shown in outline.

TABLE 2. Sequence differences observed between Lassa virus strains using partial NP gene sequences

Comparison (no. of strains)	Nucleotide difference[a]			Amino acid difference[a]		
	Range	Mean	SD	Range	Mean	SD
Among all strains (57)	0–26.8	16.2	7.7	0–14.8	7.0	4.3
Within lineage II (8)	0–17.2	13.3	5.2	0–5.7	3.8	1.8
Within lineage III with 9608911 (8)	0–23.4	13.6	6.9	0–13.4	5.2	3.5
Within lineage III without 9608911 (7)	0–16.4	10.6	5.1	0–6.2	3.4	1.9
Within lineage IV (40)	0–19.3	9.6	5.2	0–10.5	3.3	1.9
Within Guinea (5)	6.1–16.1	12.9	2.4	1.9–7.7	4.1	2.1
Within Liberia (9)	0–18.2	11.2	5.1	0–10.5	4.7	2.7
Within Sierra Leone (26)	0–12.3	4.6	3.1	0–4.8	1.7	1.1
Between lineages I and II	23.3–26.4	24.6	1.2	10.5–12.0	10.9	0.6
Between lineages I and III	23.1–25.8	24.2	1.0	10.1–13.9	11.3	1.5
Between lineages I and IV	22.0–26.4	23.5	1.0	11.0–13.9	12.0	0.6
Between lineages II and III	21.5–26.8	23.1	1.1	8.6–14.8	11.2	1.6
Between lineages II and IV	21.5–26.4	23.9	1.2	11.0–14.4	12.5	0.7
Between lineages III and IV	19.0–24.8	22.4	1.0	6.7–12.9	9.2	1.1

[a] Uncorrected p distances multiplied by 100.

Lineage IV is the largest group of Lassa virus strains in this study (Fig. 2) and contains all strains from Guinea, Liberia, and Sierra Leone (Fig. 1A). In the most parsimonious tree, strains from Guinea and Liberia occupy the basal position within the lineage with good bootstrap and BSI support for the monophyly of Lassa virus strains from Zorzor (Liberia), Kissedougou, Nzerekore, and Macenta (Guinea), and all strains from Sierra Leone. In addition, there is BSI support (three steps above parsimony) for the adjacent, distal intermediate node, which implies monophyly of strains from Zorzor, Nzerekore, Macenta, and Sierra Leone. The monophyly of all strains from Sierra Leone is well supported (84% bootstrap support; parsimony, >4 BSI) and includes the Josiah strain genetically characterized by Auperin and McCormick (5). Strains isolated from rodents are interspersed with human strains, thus establishing a genetic link between strains infecting rodents and humans in the same geographic region.

Interlineage and intralineage sequence diversity. Sequence differences within and between lineages are shown in Table 2. In this region of the NP gene, the overall genetic diversity within Lassa virus strains is great, approaching a maximum of 26.8% nucleotide and 14.8% deduced amino acid divergence. Within lineages, the diversity is generally less than 20% at the nucleotide level and 11% at the amino acid level except for lineage III when the outlier strain 9608911 is included. Within lineage IV, less variation is observed within strains from Sierra Leone than in Guinean and Liberian strains. Variation between lineages ranges from 19% to almost 27% at the nucleotide level and 6.7% to almost 15% at the amino acid level.

Lassa virus genetic distances correlate with geographic distance rather than time. Since the phylogenetic analysis revealed a relationship between geography and genetic clustering of Lassa virus strains, we proposed the hypothesis that genetic distances among Lassa virus strains correlate with geographic distance. A second hypothesis, which assumes that Lassa virus evolution is occurring at a detectable constant rate (i.e., a molecular clock), is that genetic distance correlates with time. To test these hypotheses, we chose to examine the strains from only lineage IV to avoid introducing any bias into the analysis. The great geographic distances between the origination sites of the Nigerian Lassa virus strains and the strains from lineage IV, coupled with the large "between-lineage" genetic distances, would bias the analysis toward a positive correlation between genetic distance and geographic distance. A pairwise ML genetic-distance matrix was calculated for the Lassa virus strains (36 strains) of lineage IV. Strain 807875 (Table 1), for which the site of origin was undetermined, was omitted from the analysis. A corresponding pairwise geographic-distance matrix was prepared by calculating the distance in kilometers between strain origination sites. In some instances the exact site where the patient's infection occurred was not known, and therefore the location of the hospital where the patient was evaluated was used instead. A pairwise temporal-distance matrix was prepared by counting the years separating cases. Mantel's Z statistic and Pearson's r statistic were then calculated for the genetic-geographic and genetic-temporal matrix pairs, using MatMan 1.0. The significance of the Z statistic was computed by a permutation test with 10,000 permutations. A very strong correlation between genetic distance and geographic distance was observed ($r = 0.68$; $Z = 10970.88$; $P < 0.001$). A statistically significant correlation was also observed between genetic distance and temporal distance ($r = 0.212$; $Z = 667.21$; $P < 0.05$). To rule out the possibility that the test statistic and P values were in part due to interaction between the geographic and temporal variables (i.e., genetically similar or identical strains from the same outbreak would support a correlation between genetic and both temporal and geographic variables), partial rowwise Mantel Zr and partial Pearson's r statistics were calculated using Matman with 10,000 permutations. After being statistically controlled for the corresponding geographic distance, a correlation between the genetic and temporal matrices is no longer statistically significant ($r = 0.092$; $Zr = 0.116$; $P > 0.1$). However, the correlation between the genetic-distance matrix and the geographic-distance matrix remained highly significant even after controlling for temporal distances ($r = 0.663$; $Zr = 0.341$; $P < 0.001$). Thus, the hypothesis that genetic distances among Lassa virus strains correlate with geographic distance was accepted, and the hypothesis that genetic distance correlates with time was rejected.

Lassa virus is not evolving at a detectable constant rate. While the matrix correlation analysis failed to demonstrate a correlation between genetic distance and temporal distance, we chose to further evaluate the possibility that Lassa virus evolution is occurring at a detectable constant rate (i.e., a molecular clock) by a molecular-clock likelihood ratio test. For this analysis, trees were reconstructed for all Lassa sequences in the partial NP data set (the other Old World and New World arenavirus sequences were removed), using Puzzle 4.0.1 with and without the assumption of a molecular clock. Under the molecular-clock assumption, the expected amount of evo-

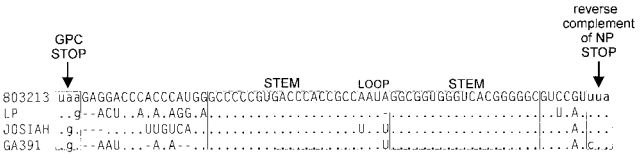

FIG. 3. Alignment of the S RNA intergenic region of Lassa virus strains 803213, LP, Josiah, and GA391. The dots indicate sequence identity with the 803213 strain. The dashes indicate alignment gaps.

lution in any lineage is proportional to elapsed time and the ML branch lengths from the root of the tree to each branch tip are the same. The QP trees were reconstructed with a HKY85 model of substitution (35), a uniform model of rate heterogeneity, 25,000 QP steps, and all parameters estimated from the data set. The LP strain of Lassa virus was selected as the root taxon based on the previous phylogenetic analysis. The tree calculated without the assumption of a molecular clock was assigned a statistically higher log likelihood score than the tree reconstructed under the assumption of a molecular clock ($-7{,}876.19$ versus $-7{,}977.38$; $P < 0.05$.) Therefore, the hypothesis that Lassa viruses are evolving in a clocklike manner was rejected.

Analysis of full-length NP and glycoprotein gene sequences. The overall topology of the most parsimonious tree suggests that the LP strain (lineage I) occupies the most basal position within the Lassa clade, followed by lineage II (southern central Nigeria). Lineages III (northern central Nigeria) and IV (Guinea, Liberia, and Sierra Leone) exhibit a sister relationship. This finding suggests that the Nigerian Lassa virus strains in lineages I and II diverged prior to strains in lineages III and IV. Though the bootstrap support for this topology is relatively weak, there is some BSI support for intermediate nodes connecting the lineages of the Lassa clade. In an attempt to better resolve these nodes, we sought to repeat the analysis using full-length NP and glycoprotein gene sequences of Lassa virus strains representative of each lineage. The NP and GPC gene sequences were already available for representatives of lineage III (GA391) and lineage IV (Josiah), so we cloned and sequenced the S RNAs of the LP strain (lineage I) and strain 803213 (lineage II) (GenBank accession numbers AF181853 and AF181854, respectively). The 5′ and 3′ noncoding regions were determined for each strain except for the 19 to 20 nt obscured by the arenavirus-specific sequences in the M13-19C primer. The full sequences of the S RNA intergenic regions were obtained for strains LP and 803213, and an alignment of these regions with those of Lassa virus strains Josiah and GA391 is shown in Fig. 3. The distribution of mutations in the intergenic region of Lassa virus S RNAs, with mutations confined to predicted single-stranded areas and absent in the regions thought to form the double-stranded stem structure, provides phylogenetic evidence for the existence of the computer-predicted stem-loop structures (7, 17).

The 1,710-nt-long NP genes of strains LP and 803213, like the NP gene of the Josiah strain of Lassa virus, lack the additional codon found at approximately nt 1132 in the NP gene of the GA391 strain of Lassa virus. In the 569-amino-acid proteins encoded by the NP gene of strains LP and 803213, the potential RNA binding sites identified by Parisi et al. (51) are completely conserved (RNP-1; zinc finger-like motif) or highly conserved (mixed-charge motif). A cytotoxic-T-lymphocyte (CTL) epitope, GVYMG, first described in the NP protein of lymphocytic choriomeningitis (LCM) virus, is completely conserved in the NP proteins of the four Lassa virus strains (residues 123 to 127) (62).

The 1,473-nt-long GPC genes of Lassa virus strains LP and 803213, like the GPC gene of strain GA391, lack the additional codon found at approximately nt 181 of the Josiah strain. Eleven predicted glycosylation sites (seven in GP1 and four in GP2) are conserved in the four Lassa strains. The region around the proposed GP1-GP2 cleavage site (14) is completely conserved, as is the proposed GP2 fusion protein sequence, GGYCLTRWMLIEAELKCFGNTAVA (28). A region in GP2, IEQQADNMITEMLQK, which contains a cross-protective Lassa virus CTL epitope (43), is also conserved.

Genetic diversity among Lassa virus strains. Sequence differences calculated using full-length Lassa virus NP, GPC, GP1, and GP2 gene and protein sequences are shown in Table 3. The NP gene and encoded protein appear to be most variable, with an average of 23.3 (range, 22.8 to 23.8) pairwise nucleotide differences and 10.2 (range, 9.5 to 12.0) amino acid differences, respectively. The full-length NP gene genetic differences correlate well with between-lineage genetic differences calculated for the partial NP gene data set (Table 2). The GP1 region is the most variable part of the GPC gene in Lassa viruses, with an average nucleotide difference of 21.6 (range, 18.9 to 24.9) and an average amino acid difference of 7.5 (range, 7.0 to 8.6). The GP2 region exhibits the least variation, especially when protein sequences are considered (Table 3). Interstrain distances are not consistent across the NP, GP1, and GP2 genes. For example, the GP2 gene of strain 803213 is most like the GP2 gene of strain GA391 at both the nucleotide and amino acid levels, but the GP1 gene sequences have the highest degree of identity with the Josiah strain. The LP strain is most unlike the GA391 strain in the protein sequence of NP and GP1 but displays the highest level of amino acid identity with GA391 in the GP2 gene product. Based on these observations, the four full-length Lassa GPC sequences were aligned and examined for evidence of recombination using RIP (38) and PLATO version 2.11 (33). No evidence of recombination among Lassa virus strains was detected, however.

TABLE 3. Sequence differences between Lassa virus strains and between Lassa strains and Mopeia virus using full-length NP and GPC gene sequences

Comparison	Difference							
	NP		GPC		GP1		GP2	
	nt[a]	aa[b]	nt	aa	nt	aa	nt	aa
Within Lassa virus								
LP(I)[c] × 803213(II)	23.8	9.7	21.0	7.4	21.1	7.4	20.7	7.3
LP(I) × Josiah(IV)	23.6	9.5	20.8	6.7	21.6	7.0	20.0	6.5
LP(I) × GA391(III)	23.3	12.0	22.4	7.4	22.2	8.6	22.6	6.0
803213(II) × Josiah(IV)	22.8	9.7	20.3	5.9	20.9	6.6	19.6	5.2
803213(II) × GA391(III)	23.3	10.5	22.1	5.7	24.9	8.1	18.9	3.5
Josiah(IV) × GA391(III)	23.2	10.0	19.1	6.7	18.9	7.4	19.5	6.0
Avg	23.3	10.2	21.0	6.6	21.6	7.5	20.2	5.8
Lassa virus × MOP[d]								
LP(I) × MOP	32.1	24.3	30.7	23.6	32.8	26.6	28.3	20.3
803213(II) × MOP	31.9	24.3	29.0	22.3	30.1	23.8	27.9	20.7
Josiah(IV) × MOP	31.7	26.0	30.5	22.7	32.2	25.7	28.6	19.4
GA391(III) × MOP	32.1	27.2	31.8	23.0	33.9	25.0	29.6	20.7
Avg	32.0	25.5	30.5	22.9	32.3	25.3	28.6	20.3

[a] Uncorrected p nucleotide distance multiplied by 100.
[b] Uncorrected p amino acid (aa) distance multiplied by 100.
[c] Lineage designation.
[d] Mopeia virus strain AN21366 (GenBank accession no. M33879).

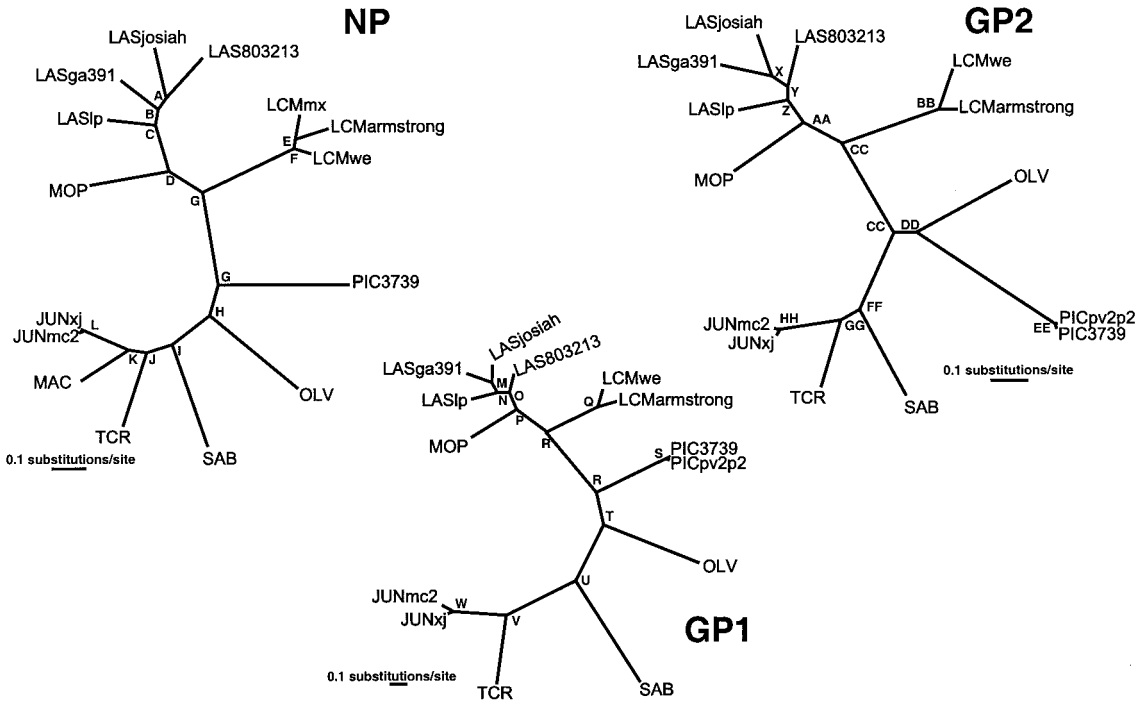

FIG. 4. Unrooted phylogenetic trees inferred using ML criteria and NP, GP1, and GP2 data sets showing relationships among arenaviruses. Full-length Lassa virus (LAS) gene sequences were aligned with the corresponding gene sequences of the Josiah (J04324 [5]) and GA391 (X52400 [17]) strains of Lassa virus, OLV virus (U34248 [9]), PIC virus strains An3739 (K02734 [6]) and PV2P2 (U77601 [64]), Junin (JUN) virus strains MC2 (D10072 [27]) and XJ (U70799 [1]), LCM virus strains Armstrong (M20869 [55]), WE (M22138 [53]), and MX (Y16308), Mopeia (MOP) virus (M33879 [63]), Tacaribe (TCR) virus (M20304 [25]), Sabia (SAB) virus (U41071 [29]), and Machupo (MAC) virus (X62616 [34]). Phylogenetic estimates were obtained by heuristic search with the HKY85 (35) substitution model and ML estimates of nucleotide frequencies, proportion of invariant sites, and transition/transversion ratios. The log likelihoods for each tree are as follows: NP, −19,134.175; GP1, −9,446.293; and GP2, −6,918.156. Branch lengths are indicated by the scale bars. The letters label nodes in reference to the bootstrap and QP reliability values shown in Table 4.

Sequence differences between Lassa virus strains and Mopeia virus. To estimate the genetic distances between Lassa virus strains and a closely related arenavirus, the genetic differences among the four Lassa virus strains and Mopeia virus strain AN21366 were calculated (Table 3.) Of the known arenaviruses, Mopeia and Mobala viruses are most closely related to Lassa viruses (11). On the basis of partial NP gene sequences, Lassa virus strains have a slightly higher degree of sequence identity (approximately 1.2%) with Mobala virus, but Mopeia virus strain AN21366 has the only full-length NP and GPC gene sequences available for comparison. Average nucleotide differences between Mopeia virus and Lassa virus strains are at least 8.4% greater than the average within-Lassa differences in comparable gene sequences and at least 14.5% higher at the amino acid level.

Phylogeny of the *Arenaviridae* estimated from NP and GP gene sequences. The NP gene sequences of Lassa virus strains were aligned with the sequences of the NP genes of other arenaviruses by using PileUp followed by manual adjustment, which ensured that the introduction of gaps maintained the open reading frame. The GPC gene sequences of arenaviruses were aligned using Clustal X version 1.63b, again with manual adjustment of nucleotide gapping guided by amino acid alignment. Heuristic searches under the ML optimality criterion were performed on the NP, GP1, and GP2 gene alignments, and the resulting phylogenies are shown in Fig. 4. Bootstrap analysis using ML was not possible for data sets of this size (14 to 15 taxa) because of the lack of computational power required to contend with such a computational effort. Topological differences are present in two areas of the trees: (i) the order of descent among the Lassa viruses differs in all three trees and (ii) Oliveros (OLV) and Pichinde (PIC) viruses are portrayed as sister taxa in the GP2 tree, whereas PIC virus has a basal position to OLV virus in the NP and GP1 trees (Fig. 4). In the NP and GP2 trees, the LP strain of Lassa virus is most ancestral, whereas 803213 occupies the basal position in the GP1 tree (Fig. 4). The Josiah and GA391 strains are portrayed as sister taxa in the GP1 and GP2 trees, but the Josiah strain exhibits a sister relationship with 803213 in the NP tree (Fig. 4).

Previous analyses of partial NP gene sequences for arenaviruses have shown that homoplasy, the similarity in character states (i.e., nucleotides or amino acid residues) for reasons other than inheritance from a common ancestor (37), manifests itself as a problem in the estimation of arenavirus phylogenies (11). Transitional saturation (the concealment of more quickly accumulating transitional substitutions by more slowly accumulating transversions over time) was very evident in partial NP gene sequence pairs exhibiting greater than 25% divergence (11). In addition, highly variable third-codon-position bases represented a source of homoplasy in phylogenetic estimates whose exclusion from analyses can dramatically change the degree of bootstrap support for some nodes (11). To remove the possible homoplastic effect of highly variable third-base positions, the ML analyses of all three genes were repeated with third-base positions excluded. Only minor changes in the inferred phylogenies were observed. In the NP tree, the branching order among the LCM virus strains was changed. In the GP1 tree, the LP and 803213 strains of Lassa virus exchanged positions so that the LP strain occupied the

most ancestral position within the Lassa clade, as in the NP and GP2 trees. In the GP2 tree, the Josiah and 803213 Lassa strains exchanged positions. Thus, the removal of third-position bases had a minimal effect on ML phylogenetic estimates for all three genes but supported the most ancestral position of the LP strain within the Lassa clade.

To compare the ML estimates with those obtained using other methods and to obtain bootstrap and reliability values, the alignments were reanalyzed using MP (both with and without third-codon-position bases), MP with a 2:1 weighting of transversions over transitions (weighted analyses and analyses excluding third-codon-position bases were performed in an attempt to remove the homoplastic influence of saturated transitions and third-codon-position bases), ME, and QP. Analyses using the MP optimality criterion were carried out as described previously for the partial NP data set, except that step matrices were incorporated into the analyses to weight transversions or assign costs to amino acid substitutions in the analysis of amino acid alignments (the weighting matrix found in the PROTPARS example nexus file was used). Analyses using the ME optimality criterion were performed by heuristic search with HKY85 distance measures (35, 42) and branch lengths constrained to be nonnegative. MP and ME bootstrap analyses analyzed 500 replicate data sets. QP analyses were carried out using the HKY85 model of substitution (35), 100,000 puzzling steps, a uniform model of rate heterogeneity, and all parameters estimated from the data set. The bootstrap (MP and ME) and reliability (QP) values for these analyses, presented in the context of how they support the ML-estimated tree topologies, are shown in Table 4. The nodes on the ML trees can be divided into three groups: (i) those supported by all analyses, (ii) those lacking support in all analyses, and (iii) those with mixed support. Nodes supported (75% or greater support) by all analyses include C, D, F, G, I, J, and L in the NP tree; O, P, Q, R, S, U, V, and W in the GP1 tree; and BB, CC, EE, FF, and HH in the GP2 tree (Fig. 4). Nodes Z and AA in the GP2 tree are supported by five of six analyses, and four of six analyses support nodes K (NP tree), M (GP1 tree), and GG (GP2 tree). Nodes lacking support in all analyses (A, E, X, and Y) are confined to regions that determine the order of descent among virus strains. Nodes that determine the order of descent among Lassa virus strains are either unsupported (A in the NP tree and X and Y in the GP2 tree) or receive mixed support. The basal position of the LP strain in the NP tree is moderately supported when MP analyses are performed without third-codon-position bases or using amino acid sequences and are strongly supported by QP analysis. In the GP1 analyses, the basal position of the 803213 strain and the sister relationship of the GA391 and Josiah strains is strongly supported by MP and weighted-MP analyses, but support decreases dramatically when third-codon-position bases are excluded or when MP analyses are performed on amino acid data. A sister relationship between OLV and PIC viruses (node DD) is proposed by the ME and QP analyses of GP2 sequences and unweighted MP analysis of NP sequences (data not shown), but the placement of PIC virus basal to OLV virus is supported by other analyses.

New World arenaviruses exhibit greater within-group genetic distances than Old World arenaviruses. In the ML trees estimated for the arenavirus NP, GP1, and GP2 data sets, the terminal branches and interspecies distances appear to be longer in the New World arenaviruses (Junin, Machupo, Tacaribe, Sabia, OLV, and PIC viruses) than in the Old World arenaviruses (Lassa, Mopeia, and LCM viruses), suggesting that the times of divergence or rates of evolution differ in the two groups. To determine if there is a significant difference in the within-group genetic distances, the ML distances between each pair of arenaviruses in the New World group and between each pair of arenaviruses in the Old World group were calculated for all three genes using nucleotide and amino acid sequence data. ML nucleotide differences were calculated using PAUP* 4.0 with the HKY85 model of substitution, and ML amino acid distances were calculated using PUZZLE 4.0.1 with the BLOSUM62 substitution model (36). Only interspecies distances were considered; interstrain distances were ignored. For all three genes, the New World arenaviruses exhibit a significantly greater mean interspecies distance than the Old World arenaviruses by *t* test for equality of means (Table 5). The significantly greater mean distance among New World arenaviruses is a function of both longer terminal-branch lengths and longer intermediate-branch lengths along the "trunk" of each tree. Since we were unable to detect clocklike evolution in the Lassa virus data set and cannot test other arenavirus species for a molecular clock because of the paucity of sequence data, we cannot determine whether the greater

TABLE 4. Bootstrap or reliability values for nodes labeled on phylogenetic trees in Fig. 4 as determined by MP, ME, and QP analyses

Gene	Node	Bootstrap or reliability value					
		MP[a]	MP, 2:1 Tv weighting[a,b]	MP, 3rd codon pos. excl.[a,c]	MP, amino acids[a]	ME[a]	QP[d]
NP	A	51	NS[e]	57	NS	NS	NS
	B	54	68	80	80	57	97
	C	100	100	100	100	100	100
	D	100	100	100	100	100	100
	E	55	55	NS	64	NS	NS
	F	100	100	100	100	92	100
	G	100	100	100	100	100	100
	H	NS	61	84	61	73	99
	I	99	99	98	98	100	100
	J	98	99	100	100	100	100
	K	66	73	95	90	100	99
	L	100	100	100	100	100	100
GP1	M	89	96	62	NS	81	77
	N	91	85	NS	54	NS	NS
	O	99	99	100	100	98	88
	P	99	96	96	100	100	94
	Q	100	100	100	100	100	100
	R	100	100	100	99	96	88
	S	100	100	100	100	100	100
	T	58	60	54	53	83	86
	U	92	97	76	98	96	99
	V	100	100	100	100	100	100
	W	100	100	100	100	100	100
GP2	X	NS	58	NS	NS	NS	NS
	Y	NS	57	70	57	NS	NS
	Z	98	98	100	100	100	68
	AA	81	96	97	71	98	90
	BB	100	100	100	100	100	100
	CC	100	100	100	100	100	99
	DD	NS	NS	NS	NS	75	85
	EE	100	100	100	100	100	87
	FF	100	100	100	100	100	100
	GG	NS	60	81	97	94	99
	HH	100	100	100	100	100	100

[a] Bootstrap values; 500 replicates.
[b] Tv, transversion.
[c] 3rd codon pos. excl., third codon position excluded.
[d] Reliability values; 100,000 puzzling steps.
[e] NS, not supported (bootstrap or reliability value, <50%).

TABLE 5. Comparison of within-group interspecies ML distances between New World and Old World arenaviruses by *t* test for equality of means[a]

Gene or protein	Distance[b]		*P* value
	NW	OW	
NP (nt)	0.633 (0.104)	0.511 (0.049)	<0.001
NP (aa)	0.658 (0.155)	0.424 (0.071)	<0.001
GP1 (nt)	1.10 (0.190)	0.588 (0.105)	<0.001
GP1 (aa)	1.66 (0.415)	0.615 (0.208)	<0.001
GP2 (nt)	0.599 (0.086)	0.434 (0.047)	<0.001
GP2 (aa)	0.595 (0.114)	0.336 (0.073)	<0.001

[a] NW, New World; OW, Old World; aa, amino acids.
[b] Mean (standard deviation).

interspecies genetic differences among the New World arenaviruses is due to a more rapid rate of evolution or a longer period of diversification.

DISCUSSION

This study represents the first large-scale examination of Lassa virus genetic variation. It shows that Lassa viruses comprise four phylogenetic lineages, three of which are found in Nigeria and the fourth in Guinea, Liberia, and Sierra Leone. An unexpected finding of the study is that the interlineage genetic distances among three Nigerian lineages (lineages I, II, and III) are greater than the distance between lineage IV (Guinea, Liberia, and Sierra Leone) and lineage III. Since this study showed a correlation between genetic and geographic distances, the high genetic diversity among Lassa virus strains within Nigeria could be attributed to the greater geographic range of the virus in Nigeria.

The finding that genetic distance among Lassa virus strains correlates with geographic distance suggests that the breeding populations of *Mastomys* sp., the rodent hosts of Lassa virus, have exhibited little regional movement since Lassa fever was first recognized in the 1960s. The lack of a correlation between genetic distance and temporal distance and the inability to detect clocklike evolution in Lassa virus strains of lineage IV indicates that the rate of genomic evolution in the NP gene of Lassa virus is low, even though the NP gene appears to be evolving faster than the glycoprotein genes. A molecular clock may be operating in Lassa virus evolution, but it cannot be detected over the relatively short time window (1969 to 1997) for which we have strain sequence data. Geographic distance may actually represent a surrogate for long-term temporal change because it would take a given unit of time for a virus to move from point A to point B, resulting from either the movement of the virus through rodent populations or the slow migration of rodent populations through geographic space. If Lassa virus sequences were recovered from retrospectively identified Lassa fever patients or rodent materials from times much earlier than 1969, perhaps from formalin-fixed materials, the molecular-clock hypothesis could be retested.

Though the order of descent of Lassa virus lineages was not fully resolved in this study, phylogenetic analysis of full-length gene sequences indicates that Nigerian strains (either LP or 803213) are ancestral to the Josiah strain from Sierra Leone. Within lineage IV, there is bootstrap and BSI support for the basal position of Guinean and Liberian Lassa virus strains relative to the strains from Sierra Leone. Both of these findings suggest a westward movement of Lassa virus from Nigeria to Guinea, Liberia, and Sierra Leone. At the moment, it is not known if the currently recognized range of Lassa fever represents part of a continuous distribution or isolated foci. There is no evidence that Lassa virus is enzootic in any of the countries separating Nigeria from Guinea, Liberia, and Sierra Leone aside from a single, serologically confirmed case of Lassa fever from Burkina Faso (61). The range of *Mastomys* sp. rodents includes all of sub-Saharan West Africa (32). Whether Lassa virus is present in *Mastomys* populations in Cote d'Ivoire, Ghana, Togo, Benin, and Burkina Faso remains to be determined.

Ter Meulen and coworkers (59) reported eight Lassa virus sequences (an NP gene region that does not overlap the partial NP gene region sequenced in this study) from Gueckedou, Guinea, that were 100% identical to the Josiah strain from Sierra Leone and one Lassa virus sequence that differed from the Josiah strain by 11.6% (nucleotide) and 10.4% (amino acid). Though we did not examine strains from Gueckedou, strains from other sites in Guinea examined in this study were quite distinct from the Josiah strain. In fact, we were unable to find another strain from Sierra Leone that demonstrated 100% identity with the Josiah strain. Therefore, we believe that the eight Guinean strains identical to the Josiah strain could possibly be the result of laboratory cross contamination. The ninth sequence probably represents an authentic Guinean Lassa virus sequence because it is consistent with the up-to-19.3 and 10.5% nucleotide and amino acid sequence divergence, respectively, noted within the strains of lineage IV.

This study examined Lassa virus strains from both *Mastomys* rodents and humans. In Sierra Leone, two *Mastomys* karyotypes have been reported (2N = 32 and 2N = 38) that are thought to represent two *Mastomys* species (52). The 2N = 32 form is thought to be either *Mastomys natalensis* or *Mastomys huberti*, and the 2N = 38 form is *Mastomys erythroleucus* (32). Lassa virus isolates have been obtained from rodents of both karyotypes (47). With the exception of strains 807875 and 808255, for which the karyotype was undetermined, all of the *Mastomys* isolates examined in this study were obtained from 2N = 32 individuals. The extent of sequence diversity for Lassa virus within each karyotype is unknown. In this study, all human strains of Lassa virus from Sierra Leone are similar to the isolates from 2N = 32 *Mastomys*, which would suggest that the two karyotypes of *Mastomys* in Sierra Leone are harboring genetically similar viruses or that the reservoir of Lassa virus infections for humans is the 2N = 32 species. This observation, however, may be due to the fact that the 2N = 32 *Mastomys* sp. processed for Lassa virus isolation is captured far more frequently in the Eastern Province of Sierra Leone than is the 2N = 38 species (47).

In this study, partial NP gene sequences were identical for five pairs of strains. Three of these strain pairs represent isolates from hospital outbreaks in which there was person-to-person transmission or infection from a common point source. Thus, the genetic sequence data obtained in this study confirm disease transmission events postulated from epidemiological observations. Strains 803201 and 803204 were isolates from an outbreak that occurred at Curran Lutheran Hospital, Zorzor, Liberia, in 1972 (50). Strain 803201 was isolated from a secondary-case patient, and 803204 was isolated from a member of the hospital staff who cared for the index case and the secondary-case patients. Strains 803213 and 803214 were isolates obtained from two missionary physicians infected at St. Borromeo Hospital, Onitsha, Nigeria, in 1974 (8). One physician was infected from the index case patient, and the second was infected while caring for the first physician after he developed Lassa fever. Strains 806319 and 806320 were isolated from two physicians infected in a hospital in Aba, Nigeria, in

1989 (22). Both had participated in a surgical procedure on the index case patient.

The epidemiological or ecological links between the last two identical strain pairs are less clear but represent isolates that originated at approximately the same time and place. Strains 803212 and 807975 were two isolates from Lassa fever patients from Vom Christian Hospital, Vom, Nigeria, in 1976. The second case, which yielded strain 803212, occurred several weeks after the first case, and it is not known if there was an epidemiological link between the two. Two identical isolates (801105 and 801107) were obtained from individual *Mastomys* females trapped in Konia village, Sierra Leone, at the identical trap site on two consecutive nights in May 1978.

At present there are no established genetic criteria for differentiating arenavirus species. The closest currently recognized genetic relationship between two accepted distinct arenavirus species is that between Junin and Machupo viruses, which exhibit 23.1 to 23.5% and 13.0 to 14.4% nucleotide and amino acid sequence divergence, respectively, in the NP gene and protein (the glycoprotein sequences of Machupo virus have yet to be reported.) The maximum amount of sequence divergence seen among full-length Lassa virus genes is 24.1% nucleotide difference (between the GP1 genes of strains 803213 and GA391) and 12.0% amino acid difference between the NP protein sequences of strains LP and GA391. Up to 21.3% nucleotide and 7.3% amino acid differences have been reported in the partial NP gene sequence of Pirital virus, a New World arenavirus (26). Others have proposed that the status of Lassa viruses as a single virus species be reevaluated only on the basis of the sequence divergence seen between the Josiah and GA391 strains (44). We believe that the range of variation seen among Lassa virus strains represents the maximum genetic variation that will be seen in a given arenavirus species. Furthermore, we propose a cutoff value of 12% amino acid difference (uncorrected p distance) in the NP protein for delineating an arenavirus species. Because mutational saturation is present in third-codon-position bases of the NP gene at genetic distances greater than 1.0, which includes all interspecies comparisons except those between Machupo and Junin viruses (data not shown), we propose that amino acid criteria be used for species definitions rather than nucleotide data. Mutational saturation is even more pronounced in glycoprotein sequences (data not shown). As more full-length glycoprotein gene sequences become available, criteria can be developed for GP1 and GP2 sequences. If the 12% amino acid cutoff for NP data (both full gene and partial gene) is used, all recognized arenavirus species would be valid except for the proposed species Pampa virus (44), which would be considered a strain of OLV virus, since there is only a 6.3% amino acid sequence divergence between partial NP protein sequences of the two viruses. Both Pampa and OLV viruses were isolated from rodents of the same genus (*Bolomys*) in Argentina (44, 48). As more intrastrain sequence data accumulate for arenaviruses, it will be possible to determine if the 12% NP amino acid cutoff remains a valid criterion for genetic species definition.

Two studies examining the relationships among arenaviruses have noted that PIC virus, or PIC and OLV viruses, cluster with the Old World viruses when glycoprotein amino acid sequences are used for phylogenetic analyses (2, 17). The studies showed dendrograms that were either midpoint rooted (17) or rooted with tospovirus (*Bunyaviridae*) gene sequences (2). We were able to reconstruct trees in which the intermediate branch connecting PIC and OLV viruses with the other New World arenaviruses is slightly longer than the intermediate branch connecting PIC and OLV viruses with the Old World arenaviruses by MP and QP analyses of translated GPC and GP1 sequences, but not GP2 sequences. The longer intermediate branch connecting PIC and OLV viruses with the other New World arenaviruses was not obtained when GP1 nucleotide data were analyzed by ML criteria (Fig. 4). Both of the previous studies (2, 17) failed to consider the effect of genetic distances within the New World and Old World groups on the rooting of arenavirus trees. We have shown in this study that the genetic distances separating New World arenaviruses are significantly greater than those within the Old World arenaviruses in the NP, GP1, and GP2 genes for both nucleotide and amino acid data. The greatest difference between mean genetic distances was observed in the GP1 gene and its protein. Thus, the majority of the total tree length is found within the New World arenaviruses. The net effect of these "lopsided" trees is that the midpoints of the GP1 and GPC trees are found in the intermediate branch separating PIC and OLV viruses from the rest of the New World arenaviruses. In most phylogenetic analyses, the use of a highly divergent outgroup tends to place the inferred ancestral node in the middle of the longest intermediate branch along the trunk of the tree. The translated glycoprotein sequence of impatiens necrotic spot virus, used as an outgroup by Albarino and coworkers (2), has less than 30% amino acid identity with the GPC protein of PIC virus even with extensive insertion of gaps into a pairwise alignment. To properly infer the ancestral node within the *Arenaviridae*, a more closely related outgroup taxon must be used. Genetic distances, in addition to cladistic relationships, must be considered also in future studies of arenavirus phylogenies.

The question arises as to whether the greater interspecies genetic differences among the New World arenaviruses compared with the Old World arenaviruses are due to a higher rate of evolution or a longer period of diversification. Studies of genomic and mitochondrial sequences have estimated the dates of evolutionary splitting of rodent lineages. For example, the split between the lineage containing the genus *Mus* (the host of LCM virus, which occupies the basal position within the Old World arenaviruses) and the lineage containing the genus *Mastomys* (the host genus of Lassa and Mopeia viruses) was estimated to have occurred 8 million years ago as determined from data obtained in genomic DNA hybridization studies (15). In studies of mitochondrial genes, Engel and coworkers (20) estimated the split of the oryzomyine lineage (containing the genus *Oryzomys*, the host genus of PIC virus, which occupies the basal position within the New World arenaviruses) from the akondontines and phyllotines at approximately 6.8 million years ago. The hypothesis that arenaviruses are coevolving with their rodent hosts has been proposed (10, 11, 16, 30, 40), and existing evidence suggests that coevolution is occurring in combination with some host-switching events (11). If arenaviruses are indeed coevolving with their rodent hosts, it suggests that the longer mean genetic distances among the New World arenaviruses compared with the Old World arenaviruses are due to a higher rate of evolution among New World arenaviruses when the rodent evolutionary time scale is applied to virus phylogenies. Estimates of the rates of evolution in arenaviruses will be necessary to confirm this hypothesis. Potential sources of "older" arenavirus sequences for estimating evolutionary rates are archived formalin-fixed clinical material and formalin-preserved rodent specimens from museum collections.

The RT-PCR primers described in this study and previous work (11) should be useful for characterizing other Lassa virus strains. The considerable genetic variation within Lassa strains, however, contributes to the potential for primer failures, as evidenced in this study by the fact that we were unable to amplify all Lassa virus strains with a single primer pair. The

method for reverse transcribing, amplifying, and cloning the complete S RNA of Lassa virus (with a few potential mismatches in the conserved 20 bases at the 5′ end) reported here is the first method described for cloning arenavirus S RNAs from a single amplicon. This technique has been used successfully to amplify and clone the S RNAs of other Old World and New World arenaviruses (data not shown) and will be a useful technique for genetically characterizing new arenaviruses, especially in cases in which internal RT-PCR primers are nonreactive.

ADDENDUM IN PROOF

Recently, Stephan Günther and colleagues at the Bernhard Nocht Institute for Tropical Medicine in Hamburg, Germany, have confirmed a Lassa virus infection in a traveler with likely exposure in Burkina Faso and Côte d'Ivoire in West Africa. Comparison of the virus nucleotide sequences which they amplified from this patient with those presented here shows the Burkina Faso/Côte d'Ivoire virus to represent an additional distinct lineage which falls between genetic lineages III and IV. These new findings further support the hypothesis that Lassa virus genetic variation correlates with geographic origin and the suggestion of a westward movement of Lassa virus from Nigeria through West Africa.

ACKNOWLEDGMENTS

We thank James Mills, Oyewale Tomori, John Krebs, and Leslie Real for helpful discussions, C. Brian Robbins for providing *Mastomys* trapping data, and Kent Wagoner for assistance with statistical analyses and map preparation.

REFERENCES

1. **Albarino, C. G., P. D. Ghiringhelli, D. M. Posik, M. E. Lozano, A. M. Ambrosio, A. Sanchez, and V. Romanowski.** 1997. Molecular characterization of attenuated Junin virus strains. J. Gen. Virol. **78:**1605–1610.
2. **Albarino, C. G., D. M. Posik, P. D. Ghiringhelli, M. E. Lozano, and V. Romanowski.** 1998. Arenavirus phylogeny: a new insight. Virus Genes **16:** 39–46.
3. **Anonymous.** 1997. Lassa fever. Wkly. Epidemiol. Rec. **20:**145–146.
4. **Auperin, D. D., M. Galinski, and D. H. Bishop.** 1984. The sequences of the N protein gene and intergenic region of the S RNA of Pichinde arenavirus. Virology **134:**208–219.
5. **Auperin, D. D., and J. B. McCormick.** 1989. Nucleotide sequence of the Lassa virus (Josiah strain) S genome RNA and amino acid sequence comparison of the N and GPC proteins to other arenaviruses. Virology **168:**421–425.
6. **Auperin, D. D., V. Romanowski, M. Galinski, and D. H. Bishop.** 1984. Sequencing studies of Pichinde arenavirus S RNA indicate a novel coding strategy, an ambisense viral S RNA. J. Virol. **52:**897–904.
7. **Auperin, D. D., D. R. Sasso, and J. B. McCormick.** 1986. Nucleotide sequence of the glycoprotein gene and intergenic region of the Lassa virus S genome RNA. Virology **154:**155–167.
8. **Bowen, G. S., O. Tomori, H. Wulff, J. Casals, A. Noonan, and W. G. Downs.** 1975. Lassa fever in Onitsha, East Central State, Nigeria in 1974. Bull. W. H. O. **52:**599–604.
9. **Bowen, M. D., C. J. Peters, J. N. Mills, and S. T. Nichol.** 1996. Oliveros virus: a novel arenavirus from Argentina. Virology **217:**362–366.
10. **Bowen, M. D., C. J. Peters, and S. T. Nichol.** 1996. The phylogeny of New World (Tacaribe complex) arenaviruses. Virology **219:**285–290.
11. **Bowen, M. D., C. J. Peters, and S. T. Nichol.** 1997. Phylogenetic analysis of the Arenaviridae: patterns of virus evolution and evidence for cospeciation between arenaviruses and their rodent hosts. Mol. Phylogenet. Evol. **8:**301–316.
12. **Bremer, K.** 1988. The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. Evolution **42:**795–803.
13. **Buckley, S. M., and J. Casals.** 1970. Lassa fever, a new virus disease of man from West Africa. 3. Isolation and characterization of the virus. Am. J. Trop. Med. Hyg. **19:**680–691.
14. **Burns, J. W., and M. J. Buchmeier.** 1993. Glycoproteins of the arenaviruses, p. 17–35. *In* M. S. Salvato (ed.), The Arenaviridae. Plenum Press, New York, N.Y.
15. **Chevret, P., L. Granjon, J.-M. DuPlantier, C. Denys, and F. M. Catzeflis.** 1994. Molecular phylogeny of the praomys complex (Rodentia: Murinae): a study based on DNA/DNA hybridization experiments. Zoolog. J. Linnean Soc. **112:**425–442.
16. **Childs, J. E., and C. J. Peters.** 1993. Ecology and epidemiology of arenaviruses and their hosts, p. 331–384. *In* M. S. Salvato (ed.), The Arenaviridae. Plenum Press, New York, N.Y.
17. **Clegg, J. C., S. M. Wilson, and J. D. Oram.** 1991. Nucleotide sequence of the S RNA of Lassa virus (Nigerian strain) and comparative analysis of arenavirus gene products. Virus Res. **18:**151–164.
18. **Clegg, J. C. S., M. D. Bowen, M. J. Buchmeier, J.-P. Gonzalez, I. S. Lukashevich, C. J. Peters, R. Rico-Hesse, and V. Romanowski.** Family *Arenaviridae*, virus taxonomy: seventh report of the International Committee on Taxonomy of Viruses, in press. Academic Press, San Diego, Calif.
19. **Djavani, M., I. S. Lukashevich, A. Sanchez, S. T. Nichol, and M. S. Salvato.** 1997. Completion of the Lassa fever virus sequence and identification of a RING finger open reading frame at the L RNA 5′ end. Virology **235:**414–418.
20. **Engel, S. R., K. M. Hogan, J. F. Taylor, and S. K. Davis.** 1998. Molecular systematics and paleobiogeography of the South American sigmodontine rodents. Mol. Biol. Evol. **15:**35–49.
21. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
22. **Fisher-Hoch, S. P., O. Tomori, A. Nasidi, G. I. Perez-Oronoz, Y. Fakile, L. Hutwagner, and J. B. McCormick.** 1995. Review of cases of nosocomial Lassa fever in Nigeria: the high price of poor medical practice. Br. Med. J. **311:**857–859.
23. **Frame, J. D.** 1975. Surveillance of Lassa fever in missionaries stationed in West Africa. Bull. W. H. O. **52:**593–598.
24. **Frame, J. D., J. M. Baldwin, Jr., D. J. Gocke, and J. M. Troup.** 1970. Lassa fever, a new virus disease of man from West Africa. I. Clinical description and pathological findings. Am. J. Trop. Med. Hyg. **19:**670–676.
25. **Franze-Fernandez, M. T., C. Zetina, S. Iapalucci, M. A. Lucero, C. Bouissou, R. Lopez, O. Rey, M. Daheli, G. N. Cohen, and M. M. Zakin.** 1987. Molecular structure and early events in the replication of Tacaribe arenavirus S RNA. Virus Res. **7:**309–324.
26. **Fulhorst, C. F., M. D. Bowen, R. A. Salas, G. Duno, A. Utrera, T. G. Ksiazek, N. M. De Manzione, E. De Miller, C. Vasquez, C. J. Peters, and R. B. Tesh.** 1999. Natural rodent host associations of Guanarito and Pirital viruses (Family Arenaviridae) in central Venezuela. Am. J. Trop. Med. Hyg. **61:**325–330.
27. **Ghiringhelli, P. D., R. V. Rivera-Pomar, M. E. Lozano, O. Grau, and V. Romanowski.** 1991. Molecular organization of Junin virus S RNA: complete nucleotide sequence, relationship with other members of the Arenaviridae and unusual secondary structures. J. Gen. Virol. **72:**2129–2141.
28. **Glushakova, S. E., I. S. Lukashevich, and L. A. Baratova.** 1990. Prediction of arenavirus fusion peptides on the basis of computer analysis of envelope protein sequences. FEBS Lett. **269:**145–147.
29. **Gonzalez, J. P., M. D. Bowen, S. T. Nichol, and R. Rico-Hesse.** 1996. Genetic characterization and phylogeny of Sabia virus, an emergent pathogen in Brazil. Virology **221:**318–324.
30. **Gonzalez, J. P., A. J. Georges, M. P. Kiley, D. M. Meunier, C. J. Peters, and J. B. McCormick.** 1986. Evolutionary biology of a Lassa virus complex. Med. Microbiol. Immunol. **175:**157–159.
31. **Gonzalez, J. P., J. B. McCormick, and M. P. Kiley.** 1988. Genetic variation among Lassa and Lassa-related arenaviruses analysed by T1-oligonucleotide mapping. Ann. Inst. Pasteur Virol. **139:**405–420.
32. **Granjon, L., J.-M. DuPlantier, J. Catalan, and J. Britton-Davidian.** 1997. Systematics of the genus Mastomys (Thomas, 1915) (Rodentia: Muridae). Belg. J. Zool. **127:**7–18.
33. **Grassly, N. C., and A. Rambaut.** 1999. PLATO: partial likelihoods assessed through optimization. University of Oxford, Oxford, United Kingdom.
34. **Griffiths, C. M., S. M. Wilson, and J. C. Clegg.** 1992. Sequence of the nucleocapsid protein gene of Machupo virus: close relationship with another South American pathogenic arenavirus, Junin. Arch. Virol. **124:**371–377.
35. **Hasegawa, M., H. Kishino, and T. Yano.** 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. **21:**160–174.
36. **Henikoff, S., and J. G. Henikoff.** 1992. Amino acid substitution matrices from protein blocks. Proc. Natl. Acad. Sci. USA **89:**10915–10919.
37. **Hillis, D. M., C. Moritz, and B. K. Mable.** 1996. Molecular systematics. Sinauer Associates, Sunderland, Mass.
38. **HIV Database and Analysis Group.** Recombinant Identification Program (RIP). Los Alamos National Laboratory, Los Alamos, N. Mex.
39. **Jahrling, P. B., J. D. Frame, S. B. Smith, and M. H. Monson.** 1985. Endemic Lassa fever in Liberia. III. Characterization of Lassa virus isolates. Trans. R. Soc. Trop. Med. Hyg. **79:**374–379.
40. **Johnson, K. M., P. A. Webb, and G. Justines.** 1973. Biology of Tacaribe-complex viruses, p. 241–258. *In* F. Lehmann-Grube (ed.), Lymphocytic choriomeningitis virus and other arenaviruses. Springer-Verlag, New York, N.Y.
41. **Keenlyside, R. A., J. B. McCormick, P. A. Webb, E. Smith, L. Elliott, and K. M. Johnson.** 1983. Case-control study of Mastomys natalensis and humans in Lassa virus-infected households in Sierra Leone. Am. J. Trop. Med. Hyg. **32:**829–837.
42. **Kishino, H., and M. Hasegawa.** 1989. Evaluation of the maximum likelihood

estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J. Mol. Evol. **29:**170–179.
43. **La Posta, V. J., D. D. Auperin, R. Kamin-Lewis, and G. A. Cole.** 1993. Cross-protection against lymphocytic choriomeningitis virus mediated by a $CD4^+$ T-cell clone specific for an envelope glycoprotein epitope of Lassa virus. J. Virol. **67:**3497–3506.
44. **Lozano, M. E., D. M. Posik, C. G. Albarino, G. Schujman, P. D. Ghiringhelli, G. Calderon, M. Sabattini, and V. Romanowski.** 1997. Characterization of arenaviruses using a family-specific primer set for RT-PCR amplification and RFLP analysis. Its potential use for detection of uncharacterized arenaviruses. Virus Res. **49:**79–89.
45. **Lukashevich, I. S., M. Djavani, K. Shapiro, A. Sanchez, E. Ravkov, S. T. Nichol, and M. S. Salvato.** 1997. The Lassa fever virus L gene: nucleotide sequence, comparison, and precipitation of a predicted 250 kDa protein with monospecific antiserum. J. Gen. Virol. **78:**547–551.
46. **McCormick, J. B.** 1987. Epidemiology and control of Lassa fever. Curr. Top. Microbiol. Immunol. **134:**69–78.
47. **McCormick, J. B., P. A. Webb, J. W. Krebs, K. M. Johnson, and E. S. Smith.** 1987. A prospective study of the epidemiology and ecology of Lassa fever. J. Infect. Dis. **155:**437–444.
48. **Mills, J. N., J. G. Barrera Oro, D. S. Bressler, J. E. Childs, R. B. Tesh, J. F. Smith, D. A. Enria, T. W. Geisbert, K. T. McKee, M. D. Bowen, C. J. Peters, and P. B. Jahrling.** 1996. Characterization of Oliveros virus, a new member of the Tacaribe complex (Arenaviridae: Arenavirus). Am. J. Trop. Med. Hyg. **54:**399–404.
49. **Monath, T. P.** 1975. Lassa fever: a review of the epidemiology and epizootiology. Bull. W. H. O. **52:**577–592.
50. **Monath, T. P., P. E. Mertens, R. Patton, C. R. Moser, J. J. Baum, L. Pinneo, G. W. Gary, and R. E. Kissling.** 1973. A hospital epidemic of Lassa fever in Zorzor, Liberia, March–April 1972. Am. J. Trop. Med. Hyg. **22:**773–779.
51. **Parisi, G., J. Echave, D. Ghiringhelli, and V. Romanowski.** 1996. Computational characterisation of potential RNA-binding sites in arenavirus nucleocapsid proteins. Virus Genes **13:**247–254.
52. **Robbins, C. B., J. W. Krebs, Jr., and K. M. Johnson.** 1983. Mastomys (Rodentia: Muridae) species distinguished by hemoglobin pattern differences. Am. J. Trop. Med. Hyg. **32:**624–630.
53. **Romanowski, V., Y. Matsuura, and D. H. Bishop.** 1985. Complete sequence of the S RNA of lymphocytic choriomeningitis virus (WE strain) compared to that of Pichinde arenavirus. Virus Res. **3:**101–114.
54. **Ruo, S. L., S. W. Mitchell, M. P. Kiley, L. F. Roumillat, S. P. Fisher-Hoch, and J. B. McCormick.** 1991. Antigenic relatedness between arenaviruses defined at the epitope level by monoclonal antibodies. J. Gen. Virol. **72:**549–555.
55. **Salvato, M., E. Shimomaye, P. Southern, and M. B. Oldstone.** 1988. Virus-lymphocyte interactions. IV. Molecular characterization of LCMV Armstrong (CTL+) small genomic segment and that of its variant, clone 13 (CTL−). Virology **164:**517–522.
56. **Southern, P. J.** 1996. *Arenaviridae*: the viruses and their replication, p. 1505–1519. *In* B. N. Fields, D. M. Knipe, P. M. Howley, et al. (ed.), Fields virology. Lippencott-Raven, Philadelphia, Pa.
57. **Strimmer, K., and A. von Haeseler.** 1997. PUZZLE. Zoologisches Institut, Universität München, Munich, Germany.
58. **Swofford, D. L.** 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland, Mass.
59. **Ter Meulen, J., K. Koulemou, T. Wittekindt, K. Windisch, S. Strigl, S. Conde, and H. Schmitz.** 1998. Detection of Lassa virus antinucleoprotein immunoglobulin G (IgG) and IgM antibodies by a simple recombinant immunoblot assay for field use. J. Clin. Microbiol. **36:**3143–3148.
60. **Ter Meulen, J., I. Lukashevich, K. Sidibe, A. Inapogui, M. Marx, A. Dorlemann, M. L. Yansane, K. Koulemou, J. Chang-Claude, and H. Schmitz.** 1996. Hunting of peridomestic rodents and consumption of their meat as possible risk factors for rodent-to-human transmission of Lassa virus in the Republic of Guinea. Am. J. Trop. Med. Hyg. **55:**661–666.
61. **van der Heide, R. M.** A patient with Lassa fever from the Upper Volta, diagnosed in the Netherlands. Ned. Tijdschr. Geneeskd. **126:**566–569. (In Dutch.)
62. **Whitton, J. L., A. Tishon, H. Lewicki, J. Gebhard, T. Cook, M. Salvato, E. Joly, and M. B. Oldstone.** 1989. Molecular analyses of a five-amino-acid cytotoxic T-lymphocyte (CTL) epitope: an immunodominant region which induces nonreciprocal CTL cross-reactivity. J. Virol. **63:**4303–4310.
63. **Wilson, S. M., and J. C. S. Clegg.** 1991. Sequence analysis of the S RNA of the African arenavirus Mopeia: an unusual secondary structure feature in the intergenic region. Virology **180:**543–552.
64. **Zhang, L., K. Marriott, and J. F. Aronson.** 1999. Sequence analysis of the small RNA segment of guinea pig-passaged Pichinde virus variants. Am. J. Trop. Med. Hyg. **61:**220–225.
65. **Zweighaft, R. M., D. W. Fraser, M. A. Hattwick, W. G. Winkler, W. C. Jordan, M. Alter, M. Wolfe, H. Wulff, and K. M. Johnson.** 1977. Lassa fever: response to an imported case. N. Engl. J. Med. **297:**803–807.